The New, Third-generation, AC-DC Electrical Penetration Graph (EPG) Monitor and Its Usefulness for IPM Research on Vectors of Plant Pathogens

Elaine Athene Backus[1, 2, a]

[1] USDA Agricultural Research Service, San Joaquin Valley Agricultural Sciences Center, 9611 So.Riverbend Ave, Parlier, CA 93648 USA
[2] Corresponding author, E-mail:Elaine.Backus@ARS.USDA.gov
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ABSTRACT

The most rigorous method to identify feeding behaviors of hemipteran vectors of plant pathogens is electrical penetration graph (EPG) monitoring. The purpose of this talk was to review: 1) principals of EPG as a tool for developing novel integrated pest management tools against vectors, and 2) application of EPG to identify feeding behaviors leading to inoculation of *Xylella fastidiosa*. *X. fastidiosa* is a xylem-limited bacterium that causes several scorch diseases in important crops, such as Pierce's disease of grape. Bacteria form a dense biofilm on the foregut cuticle of the glassy-winged sharpshooter, *Homalodisca vitripennis* (Germar) and other xylem-feeding vectors. Bacteria are inoculated directly from sites in the foregut into a host plant during sharpshooter feeding (i.e. probing of the mouthparts, stylets, into the plant). However, despite nearly 70 years of research, no one had associated specific sharpshooter stylet probing behaviors with inoculation until EPG was employed for such research. Development of the third generation (AC-DC) EPG monitor from the first two generations of monitors (AC and DC) helped define the mechanism of *X. fastidiosa* inoculation. EPG and other evidence for the salivation-egestion hypothesis for *X. fastidiosa* inoculation, in which salivation combined with egestion [outward fluid flow] carries bacteria into the xylem, was reviewed. Understanding the inoculation mechanism will aid development of grape varieties resistant to inoculation of *X. fastidiosa* by sharpshooter vectors.

Keywords: Hemiptera, Electrical penetration graph, EPG, feeding, vector, *Xylella fastidiosa*

INTRODUCTION

Studying the feeding, plant damage, and (especially) transmission (i.e. acquisition, retention and inoculation) of plant pathogens by hemipteran insect pests has always been a challenge. This is because their specialized, piercing-sucking mouthparts, the stylets, are probed/penetrated into opaque plant tissues through which the stylets cannot be directly, visually observed during probing. Early studies of hemipteran feeding were forced (by the technical limitations of their time) to study snapshots of feeding, after the fact. For example, one could view deposits of hardened saliva *in planta* left behind after feeding activities had ceased [22], in essence, frozen in time. Or, one could quantify collections of excretory droplets [18] or document transmission of plant pathogens from long-past bouts of feeding [4,29]. However, a researcher could not directly study or quantify hemipteran feeding in real time, as it was occurring, until the invention of are volutionary technology nearly 50 years ago. That technology is electrical penetration graph (EPG) monitoring of insect feeding[3,13,35].

Today, EPG is used in three main ways for development of novel integrated pest management (IPM) tactics for hemipteran pests, especially vectors of plant pathogens. First, in cases where the fundamental mechanisms of feeding damage or transmission of a plant pathogen are unknown, EPG is instrumental in elucidating such information. Second, once such mechanisms are understood, EPG can be used to demonstrate the effects of insecticides, antifeedants, or other chemical compounds on specific feeding behaviors responsible for pathogen transmission. Third, EPG can similarly identify the effects of resistant vs. susceptible varieties of crop plants, including those genetically engineered to express biopesticides. Rapid computerized analysis of EPG data can provide quantitative comparisons of, for example, the responses of vectors to resistant and susceptible plants. A researcher can then predict whether a pathogen will be transmitted from/to a putatively resistant host plant, leading to a novel mechanism for host plant resistance.

The purpose of this paper is to review: 1) the principals and history of EPG, especially development of the new, third-generation AC-DC monitor, and 2), a summary of my own work on the mechanisms of transmission of *Xylella fastidiosa* by sharpshooter leafhoppers. Much of the writing herein is excerpted and/or adapted from Backus [7], to which the reader is referred for more information.

Principles and history of EPG monitoring of insect feeding

Most advances in understanding the role of vector feeding behavior in mechanisms of plant pathogen transmission have been made possible by use of EPG over the last 50 years. As diagrammed in Fig. 1a, the insect is made a part of an electrical circuit by attaching a thin (~10-60 μm) gold wire to its dorsal surface with conductive glue, then connecting the insect to the input of a head stage amplifier attached to a monitor that also electrifies the plant. When the insect's stylets probe the plant tissues, fluids in the stylet food and salivary canals ionically conduct the electrical signal through the insect to the monitor, where it is amplified and outputted to a computerized digital display. Variable biopotentials (i.e., biological voltages) and/or electrical resistances to fluid flow (Fig. 1b) generated by the insect-plant interface instantaneously transform the constant applied signal into a variable-voltage output signal that is graphed as a waveform. The biological meanings of EPG waveforms are defined by correlation with: 1) stylet tip locations in the plant, via histology of salivary sheaths or cut stylets in probed plant tissues, and 2) intricate stylet activities (e.g. stylet movements, salivation, etc.) performed by the insect, via observation in transparent artificial diets and other methods [35]. Electrical resistance and biopotentials each produce different parts of the waveforms, depending on the generation of EPG technology used.

First developed in the late 1950's to early 1960's, EPG has advanced over the last 50 years along with the revolution in electronics. The earliest, first-generation EPG monitors, developed by McLean and Kinsey [23], used technology typical of the time, i.e., glass-tube amplifiers in the late 1950's, later evolving into early solid-state transistors by the 1960's. They also used AC (alternating current) applied signal, and a low amplifier sensitivity or input impedance (Ri) of 10^6 Ohms[24]. Monitors with low Ri outputted signals caused primarily by electrical resistance to/conductance of ionic charges carried in fluids (e.g. saliva, plant fluids) passing through the stylets (modeled as a variable resistor, Ra, in Fig. 1b) [35]. Thus, the earliest AC monitors best detected information such as starting and ending of stylet probing, saliva secretion and salivary sheath formation (today termed pathway activities), stylet movements such as extension, retraction, and partial stylet withdrawal, and stylet contact with vascular tissues such as phloem and xylem[13].

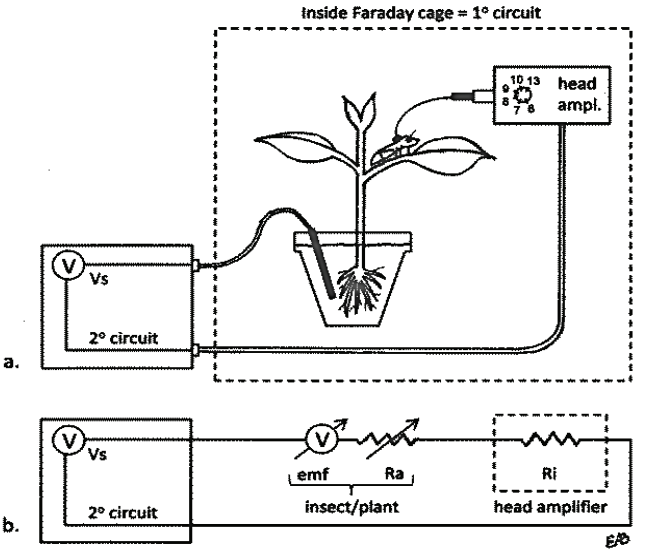

Fig. 1. Diagrammatic representation of the primary (1^o) circuit of an EPG monitor. **a.** Realistic model of the plant and insect. **b.** Electronic block diagram of primary circuit, including variable biopotentials (emf) and variable resistance (Ra). Modified from an original drawing by G. P. Walker [35]. 2^o = secondary circuit (i.e., signal processing circuitry); head ampl. = head stage amplifier; emf = electromotive force (biopotential); Ra = insect (e.g., aphid) resistance; Ri = input impedance of the head amplifier; Vs = source voltage. (Reprinted with permission of the American Phytopathological Society.)

By the late 1970's, electronics had been revolutionized with improved solid-state transistor technology (operational amplifiers, or op amps), so that more sophisticated amplifiers and recording devices were available and affordable. The second-generation (termed DC) monitor, developed by Tjallingii [33], used op amps in simple printed circuits, DC (direct current) applied signal, Faraday cages to control noise, FM tape recorders or rapid-response strip chart recorders as output devices, and (most importantly) higher amplifier sensitivity (Ri of either 10^9 or 10^{13} Ohms) [33; 34]. Tjallingii [33; 34] also established the modern theoretical understanding for EPG science by introducing the concepts of the R (or resistance) component (Ra, described above) and the electromotive force, emf(synonymous with biological voltage orbiopotential) component, blended together in the output signal [35]. The R and emf components are also termed electrical origins of a waveform. There are two known mechanisms underlying the emf component in the plant or plant-insect interface. The first is

disruption of the plant cell membrane's charge separation between external and internal cell environments, by stylet tips breaking living plant cell membranes. Such membrane breakages lead to two electrical effects on waveform output: 1) sudden voltage drops as the stylets puncture a membrane, and 2) positive vs. negative voltage levels that indicate extracellular (apoplastic) vs. intracellular (symplastic) stylet tip positions, respectively. The second biopotential mechanism is streaming potentials, i.e., voltages developed by charge separation that occurs nearly instantaneously in ionic fluids rapidly moving through thin capillary tubes, such as the stylet food and salivary canals [35]. Streaming potentials cause regular-frequency waveforms that result from rhythmic pumping of muscles [18; 35]. Increased amplifier sensitivity in the DC monitor made it possible to detect both R components (previously detected by the AC monitor) and additional emf components in the EPG output waveform. Identifying the electrical origin(s) of a waveform greatly aided in defining its biological meaning.

Another valuable theory of Tjallingii for sharpshooter studies was the sigmoidal R/emf responsiveness curve [33, 34], produced when the proportion (0-100%) of emf in an insect's total EPG output signal is graphed in relation to Ri level (on a scale of 10^6 to 10^{13} Ω) [11]. The lower the Ri level, the smaller the proportion of the total signal that consists of emf (Fig. 2). Because the ratio of R:emf is reciprocal within the total EPG signal (system voltage), the smaller the emf proportion, the larger the R proportion [11,12]. In addition, the position of the R/emf responsiveness curve with respect to Ri can shift with the size of the insect being recorded (Fig. 2; compare small aphids vs. small leafhoppers vs. sharpshooters). Generally, the larger an insect's body (i.e.,with larger-diameter food and salivary canals, therefore greater ionic conductivity), the more the responsiveness curve shifts towards the left on the chart (Fig. 2). Thus, lower Ri levels allow detection of more emf in the signal of large hemipterans like sharpshooter leafhoppers, compared with smaller species like aphids. The maximum number of waveforms, and thus of detectable probing behaviors, will be detected at the 50:50 R:emf balance point. Tjallingii chose an intermediate Ri level of 10^9 Ohms for his (DC monitor) design, to balance R and emf components for small aphids. Thus, the R/emf responsiveness curve explains why an AC monitor (Ri 10^6 Ohms) detects almost no emf component in aphid probing [11], but slightly more in sharpshooter waveforms [13].

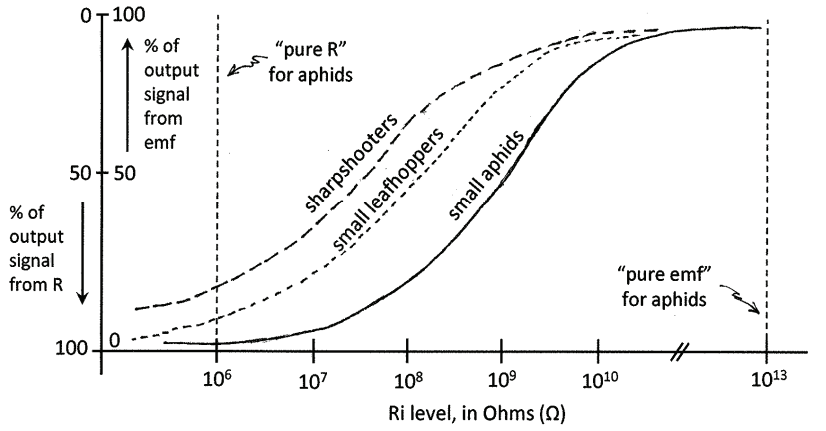

Fig. 2. Theoretical R/emf responsiveness curves for selected example insects. See text for summary, and [11] for more detailed explanation of theory. (Reprinted with permission of the American Phytopathological Society.)

By the mid-1990's, all EPG researchers were using computerized analog-to-digital waveform display, greatly improving waveform fine-structure detail from both AC and DC recordings [31]. Also by that time, EPG had gradually become specialized so that AC monitors were used primarily for medium-to-large insects such as leafhoppers, planthoppers, and other auchenorrhynchans, while DC monitors were used for smaller insects, especially aphids, whiteflies, thrips, psyllids, and other sternorrynchans [5]. In retrospect, the R/emf responsiveness curve explains part of the success of such recordings, because signals from small insects like sternorrhynchans would contain both R and emf at Ri 10^9 Ohms, while recordings of larger insects like auchenorrhynchans would contain both components at lower input impedance (Ri 10^6 Ohms). However, there also may be a difference in tolerance to type of applied signal. Although such differential tolerance is poorly known and an active area of research, anecdotal observations suggest that some leafhoppers (especially sharpshooters) seem to tolerate AC better than DC at different Ri levels, while aphids may tolerate DC better (Backus *unpub. data*). To increase flexibility for all types and sizes of insects, a third-generation EPG monitor (termed AC-DC) was developed by Backus and Bennett [11]. It provides selectable Ri levels of 10^6-10^{10} plus 10^{13} Ohms, choice of AC or DC applied signal, and modernized, up-to-date electronics such as instrumentation-quality op amps on standardized, commercially-manufactured printed circuit boards. This instrument removes artifactual voltages and other problems of older electronics [11, 12]. In addition,

the AC-DC monitor was specifically designed to incorporate all functions of the second-generation DC monitor, while also expanding its capabilities to include greater flexibility of settings. Thus, a researcher can tailor the monitor settings to the specific needs of each insect species recorded. As a result, the AC-DC monitor combines all the advantages of both previous generations of EPG monitor, with none of the disadvantages. Using the AC-DC monitor, it was found that there were no differences in appearance of aphid waveforms based on type of applied signal *per se*, i.e., AC vs. DC. However, large differences occurred based on Ri level, as predicted by the R/emf model [(11)].

Background on *Xylella fastidiosa* and its vectors, sharpshooter leafhoppers

An intimate understanding of the transmission mechanism of *Xylella fastidiosa* (Wells), causative agent of Pierce's disease of grape and numerous other scorch diseases, has eluded scientists for nearly 70 years. The transmission mechanism of *X. fastidiosa* is unique, because the bacterium is the only known arthropod-transmitted plant pathogen that is non-circulative yet also propagative in its vector. Bacteria are acquired and grow as a complex biofilm on the cuticle of the functional anterior foregut in its vectors. Thus, the pathogen is considered foregut-borne [(27)]. The best-studied vectors of *X. fastidiosa* are sharpshooters, comprising two tribes (Cicadellini and Proconiini) in a subfamily (Cicadellinae) of leafhoppers (Cicadellidae) within the suborder Auchenorrhyncha, order Hemiptera.

Bacterial cells are inoculated directly into the plant during sharpshooter stylet probing into xylem vessels. Yet, exactly how such reverse-flow of fluid out of the stylets is possible, and exactly what probing behaviors are responsible, has been an intractable problem until recently. Probing behaviors of salivary sheath-making hemipteran insects like sharpshooters are quite complicated. Therefore, it has been a challenge to answer the "essential question" [(2)]about *X. fastidiosa* inoculation into plants, i.e., *what (specific) probing behaviors are associated with inoculation*? EPG has been vital for the solution.

The anterior foregut in sharpshooters (Fig. 3) consists of two parts; the first is a narrow channel, the precibarium [(15)] that conveys fluid from the food canal in the stylets to the second part, the sucking pump or cibarium. Lining the precibarium are two groups of gustatory (taste) chemosensilla separated by a small, flap-like valve. The precibarial valve occurs in a basin whose structure suggests that fluid is channeled to force the valve

to passively close [15]. Yet, the valve is also powered by a tiny muscle (Fig. 3, vm) whose innervation and location are independent of the cibarial dilator muscles (Fig. 3) that lift the lid of the pump, called thecibarial diaphragm[16]. Fluid is swallowed from the cibarium through the true mouth (Fig. 1, *) into the true foregut, composed of the narrow,short pharynx, then the wider, longer esophagus. The end of the foregut occurs at the non-muscular, passively closing esophageal valve at the entrance to the midgut. The esophageal valve functions in all hemipterans to prevent regurgitation, defined as movement of fluid from the midgut "backwards" into the foregut [19]. Fluid outflow from the stylet tips was thought impossible due to the esophageal valve, until Harris [20] found evidence of (and named) 'egestion' in aphids, and Backus [6] proposed that the egested fluid originated in the precibarium.

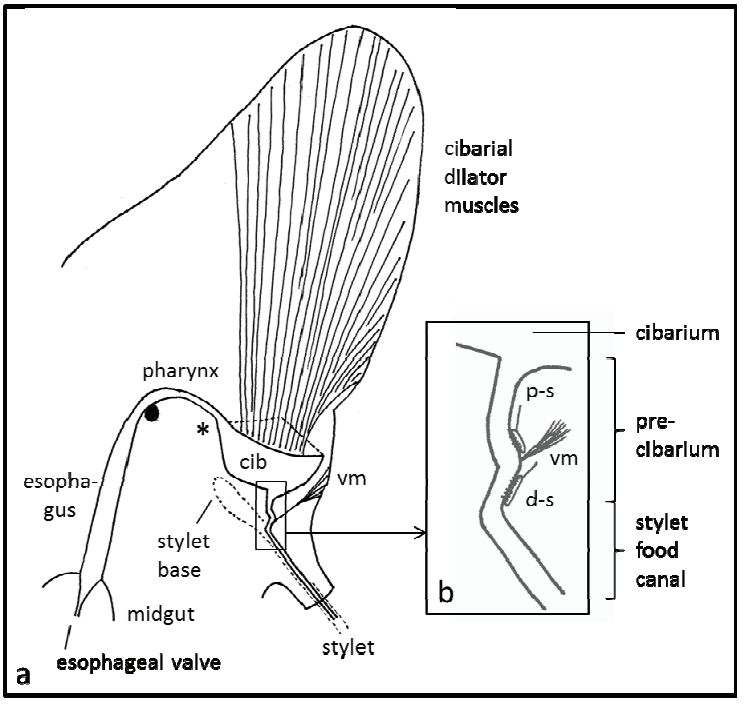

Fig. 3. a. Side view of the head of *H. vitripennis*showing the sizes and positions of structures (described in the text) in the functional foregut of sharpshooters. Small box near the cibarium (cib) is enlarged in inset, part b. *, location of the true mouth; vm, precibarial valve muscle. **b.** Enlargement of the boxed area in part a, showing the fluid-conducting channel formed by the convergence of the stylet food canal, precibarium and cibarium. d-s, neuron cell bodies and nerve from the D (distal)-sensilla; p-s, cell bodies and nerve from P (proximal)-sensilla; black circle, cross section of the tentorium. (Reprinted with permission of the American Phytopathological Society.)

EPG monitors used for studies of sharpshooters

Three of the four EPG-studied sharpshooter species are North American: the glassy-winged sharpshooter, *Homalodisca vitripennis* (Germar) (formerly *H. coagulata*) [32], the smoke tree sharpshooter, *H. literata* Ball, and the blue-green sharpshooter, *Graphocephala atropunctata* (Signoret). The fourth is a tropical Brazilian species, *Bucephalogonia xanthophis* (Berg). The *Homalodisca* spp. are in tribe Proconiini, while *G. atropunctata* and *B. xanthophis* are in tribe Cicadellini.

Seven EPG studies of sharpshooter feeding have been published. *G. atropunctata* was recorded with an electronically updated first-generation (AC) monitor (Ri of 10^6 Ohms) with dynamic noise cancellation [3,10], *B. xanthophis* was recorded with a second-generation (DC) monitor (Ri of 10^9 Ohms) [25], and *Homalodisca* spp. were recorded with a third-generation (AC-DC) monitor (at all Ri levels, but especially 10^6-10^9 Ohms) [7]. AC-DC recordings provided a means of cross-referencing the recordings of the older AC and DC monitors; they revealed waveforms for *Homalodisca* spp. that were nearly identical to those of *G. Atropunctata* and *B. xanthophis* when AC-DCRi levels were set to the same as used for the latter two species, i.e., 10^6 and 10^9 Ohms, respectively. Most importantly, intermediate Ri levels of 10^7 and 10^8 Ohms outputted waveforms intermediate in appearance between older AC and DC monitors. Thus, as predicted, AC-DC recordings bridged the large difference in waveform appearances between the earlier types of recordings of sharpshooters, as also observed for aphid EPG recordings [11].

Mechanism of inoculation of *Xylella fastidiosa* by sharpshooters

EPG evidence reviewed in more detail elsewhere [7] describes experiments that defined the biological meanings of four of the component waveforms of the sharpshooter X wave, i.e., B1w, fB1w, B1s, and C1, showing that they represent the salivation and egestion behaviors of vectors [14]. Based on this EPG evidence, the salivation-egestion hypothesis was proposed. Briefly, it states that inoculation of *X. fastidiosa* occurs when saliva taken up into foregut is egested (via two different mechanisms, rinsing and discharging egestion) into the plant, carrying bacteria.

In more detail, inoculation begins when still-liquid enzymatic gelling saliva[9] is secreted into the plant (represented by B1w), at any point in stylet probing but especially when the stylet tips are in a xylem cell. A portion of that saliva is rapidly brought into the precibarium (via cibarial diaphragm quivering during fB1w; Fig. 4a and inset boxes),

where bacterial cells have already been acquired and colonized. Fluid in the precibarium is then swished back and forth across the distal and proximal precibarial chemosensilla by fluttering of the precibarial valve (during B1s), located between the two sets of sensilla (Fig. 4b). Hypothesized enzymatic degradation by saliva of the matrix cementing *X. fastidiosa*cells to the cuticle [9], combined with mechanical rupture, would cause some cells to be dislodged. Once gustatory sampling is completed, this fluid is not swallowed [14], but instead is swept out through the stylet tipsvia rinsing egestion [17].

Rinsing egestion occurs when the precibarial valve is fully functional, i.e., its rapid movement sweeps material from distal to (below) the valve out of the stylets. Although the precibarium had been proposed as the "staging area" in the foregut from which *X. fastidiosa* bacterial cells are egested [4], the manner and exact precibarial site from which material is egested were not known until a 2011 study [17]. Populations of bacteria in the cibarium remained in equilibrium over six days of acquisition access period (AAP) (Fig 5), when imaged daily[17]. In contrast, populations in the precibarium increased in a progressively more distal (lower) direction from the cibarium, every two to three days. It was striking that virtually no bacterial colonization (only 7 of 50 insects) occurred distal to the valve, and then only when space above (proximal to) the valve was already colonized (e.g., Fig. 5, Day 3). Lack of bacterial biofilm distal to the precibarial valve was interpreted as evidence of fluid turbulence due to valve fluttering, effectively preventing bacterial attachment under normal circumstances. As long as the valve could move, any microbes growing or carried into the area below it would be swept into the food canal and out the stylet tips.

Waveform B1s, and therefore rinsing egestion, is performed in all cell types along the stylet pathway as well as in the xylem [14]. Only a small number of *X. fastidiosa* cells would be rinsed from the area distal to the valve, because the area is small compared with the total length of the precibarium. This explains why small numbers of *X. fastidiosa* have been detected outside of xylem in sheath saliva deposited by *H. vitripennis* on glass [30], in artificial diet [1], and in varying lengths of salivary sheaths in all probed plant tissues (Backus, unpub. data). The presence of bacterial cells in saliva supports the salivation-egestion hypothesis [14]. Symptomatic consequences of inoculation into non-xylem cells are presently unknown, although it is assumed that bacteria cannot survive outside xylem.

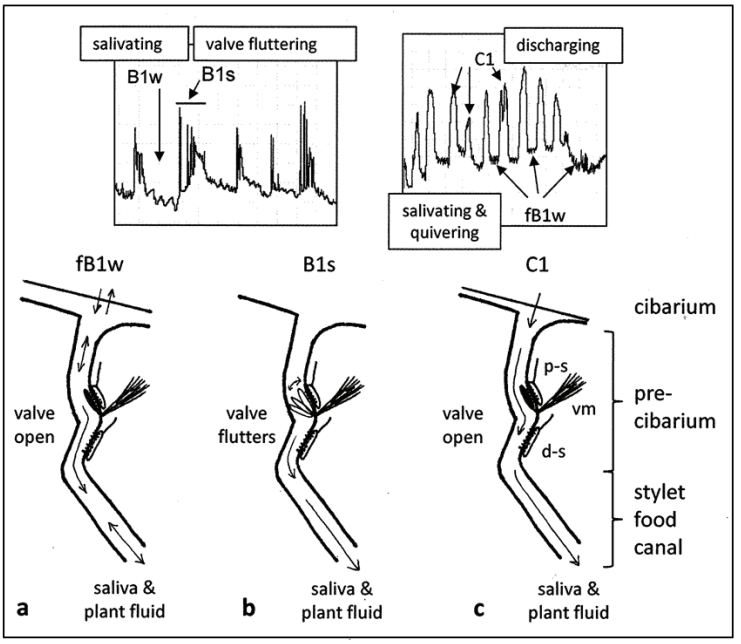

Fig. 4. Illustration of the sequential behavioral steps represented by the parts (inset boxes) of the sharpshooter X wave. **a.** Combined salivation and cibarial quivering during fB1w pulls small amounts of saliva plus plant fluid into and out of the precibarium while the precibarial valve (black oval) is open. **b.** Fluttering of unencumbered precibarial valve (black-lined oval) (and possibly also cibarial quivering, not shown) during B1s causes fluid turbulence and outward push of fluid below (distal to) the valve (rinsing egestion). **c.** Rapid fall of the cibarial diaphragm during C1 causes strong flow of fluid outward (discharging egestion) that may dislodge blockages clogging the valve (black oval) open. d-s = distal (precibarial chemo)sensilla; p-s = proximal sensilla; vm = valve muscle. (Reprinted with permission of the American Phytopathological Society.)

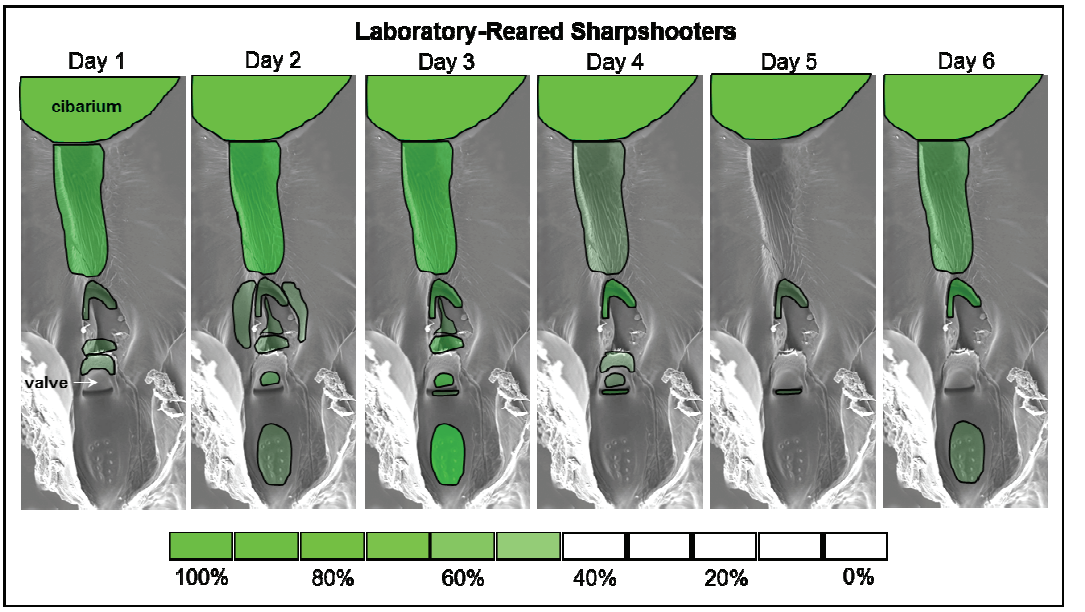

Fig. 5. Pictorial representation of the percentage of 10 laboratory-reared sharpshooters for each of 6 days of AAP that showed any amount of *X. fastidiosa* bacteria in each location in the precibarium or cibarium. Data are visually represented by green shapes superimposed over a scanning electron micrograph of a clean precibarium, as an anatomical reference. Each shape represents one of nine different locations in the precibarium. Degree of transparency of shape denotes percentage, as shown in the key on the bottom. For example, the most opaque area (the cibarium) had a probability of 100% bacterial occupancy (10/10 insects), whereas the most transparent green area (under the precibarial valve, day 4) had a probability of 10% occupancy. Modified from [17]; for names of precibarial locations, see original figure. (Reprinted with permission of the American Phytopathological Society.)

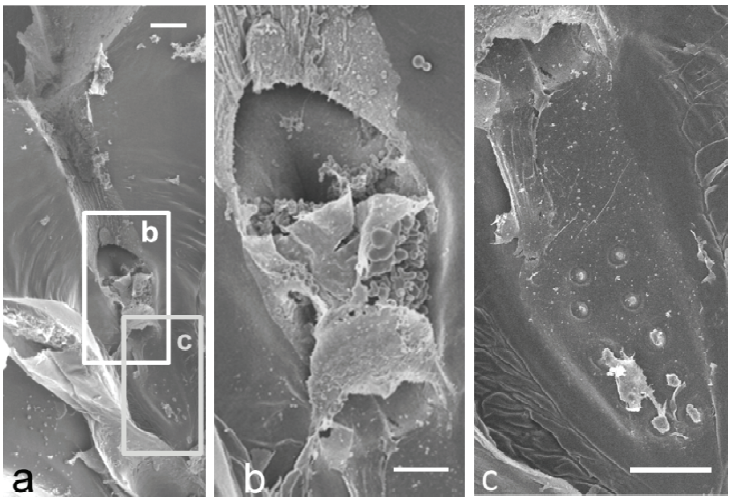

Fig. 6. Scanning electron micrographs of microbes on the floor of the precibarium of field-collected *H. vitripennis*. **A.** Overview, showing microbial biofilm in the cibarium that has grown into the trough of the precibarium. Boxes b and c are enlarged in parts b and c. Scale bar, 25 μm .**b.** Close-up of unknown, rounded microbes in biofilm matrix that overlays the precibarial valve and part of the basin around it; the valve appears to be affixed open by the accumulated biofilm. Scale bar, 10 μm. **c.** Close-up of the D sensilla field distal to the precibarial valve showing biofilm deposits extending from the affixed valve, and encroaching on the D-sensilla. Scale bar same as part b. (Reprinted with permission of the American Phytopathological Society.

Discharging egestion occurs when accumulations of microbes (either *X. fastidiosa* or some other microbial species) cover or clog the precibarial valve, causing it to be affixed open (Fig. 6). Such valve malfunction would likely cause fluid turbulence in the distal end of the channel to be greatly diminished, and allow microbial colonization to occur below the valve. Once the full length of the precibarium becomes heavily colonized by microbes (e.g., Fig. 5, Day 3), the insect must clear such obstructions; otherwise, it will be unable to taste its food or swallow. The only way to mechanically scrub away obstructing material in the precibarium would be sudden, rapid (snap-like) release of the cibarial diaphragm, before the cibarium is completely full. Such rapid cibarial release has been correlated with waveform C1 [(7)]. Rapid drop would forcefully propel fluid into the precibarium and out the stylets (Fig. 7c); this propulsion is termed discharging egestion.The sudden disappearance of all *X. fastidiosa* accumulations along the full length of the precibarium on the fourth to fifth days of AAP (Fig. 5, Day 5) provides evidence of discharging egestion from the precibarium [(17)]. If they were being swallowed, the bacteria would not disappear from below (distal to) the precibarial valve.

Also, the density of bacteria in the cibarium, as well as precibarium, would significantly (and simultaneously) decrease; yet the cibarium remains fully occupied (Fig. 5).

Discharging egestion is probably the most epidemiologically significant inoculation behavior performed by sharpshooter vectors. Based on the above EPG evidence, it is proposed that discharging egestion occurs nearly exclusively in xylem cells [14]. If a vector's precibarium is heavily loaded with *X. fastidiosa* cells, stronger and more frequent events of discharging egestion must be performed within multiple xylem cells, both rejected and accepted cells [14]. Accordingly, the largest number of *X. fastidiosa* cells would be inoculated into the largest number of xylem cells from the most heavily clogged precibaria, probably every two to three days. This may be one reason why AAPs lasting at least two days in source plants have the highest single-probe success of inoculation[21] (Backus, unpub. data). It also supports previous findings that success of acquisition in large part determines success of inoculation [6].

When a sharpshooter secretes and then takes up saliva into its precibarium during the X wave, the bulk of material egested will be saliva, and thus any egested bacteria may be embedded in a bolus of (probably hardened) sheath saliva in the xylem, such as that shown in Fig. 7a. Would such bacteria be permanently entrapped, or can they escape from the saliva to initiate the infection process? To begin to address this question, a small study was performed to examine the interaction between bacteria and saliva in grapevine petioles [8]. Eight *H. vitripennis* were restricted to a 5 cm length of stem for 24 hrs, to produce numerous salivary sheaths in the stem. After removal of insects, green fluorescent protein-expressing *X. fastidiosa* (*Xf* GFP) were mechanically inoculated into the same area; tissues from the insect-probed, inoculated stems were excised 30-60 min later, prepared for immunohistology, and 10 salivary sheaths near needle inoculations of bacteria were examined via confocal microscopy. Four of the ten sheaths had entered xylem vessels that later became filled with *Xf* GFP transported from distant needle punctures. Thus, there was no physical needle-damage to the hardened sheath saliva therein. Most bacteria in the vessels were clearly outside the sheath saliva (Fig. 7a and b). However, a small number were inside the saliva, near the edge of the bolus (Fig. 7b). Several lines of evidence [8] support that the *Xf* GFP were able to penetrate into the portion of hardened saliva left by feeding sharpshooters in the xylem vessel, by some unknown mechanism, perhaps bacterial secretion of enzymes. Such penetration into semi-solid enzymatic gelling saliva suggests that *X. fastidiosa* should be able to move out of soft, newly-secreted saliva before it had fully stiffened, thus providing indirect

evidence for the salivation-egestion hypothesis.

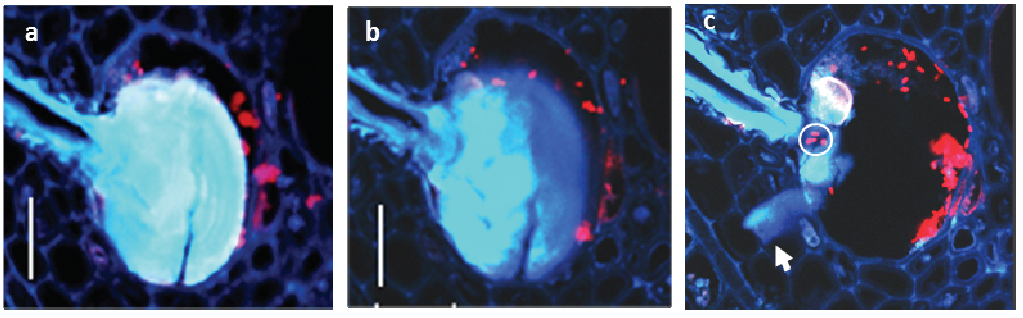

Fig. 7. Confocal microscopy image of a bolus of gelling saliva (pale teal blue) injected into a grapevine stem xylem cell at the end of a probe by *H. vitripennis*, several hours prior to mechanical inoculation of *X. fastidiosa* into the same stem. Inoculated bacteria (red) were translocated upward in xylem into the vicinity of this previously secreted, undamaged salivary sheath. **a.** Full, 10 µm-thick section, showing immunostained bacteria in the xylem cell, but outside the salivary bolus. **b.** An optical section 0.5 µm thick, through the middle of the same salivary bolus, showing red-immuno-stained bacteria inside the outer edge of the bolus (arrows). **c.** An optical section 0.5 µm thick, through the side of the same salivary bolus, showing three red-immunostained bacteria (white circle), possibly wild-type, near the stylet entry point. All three images are the same magnification; both scale bars 25 µm. (Reprinted with permission of the California Department of Food and Agriculture.)

In addition, the immunohistology study of *H. vitripennis*-probed grape stems described above detected three *X. fastidiosa* cells embedded in the salivary bolus near what appears to be the stylet entry point, far from the bacteria that penetrated the exterior of the bolus (Fig. 7c) (8). These cells may have been wild-type bacteria (also immunostained, similar to the mechanically inoculated bacteria) injected into the xylem vessel by the sharpshooter, known to have acquired *X. fastidiosa*. This finding also supports the salivation-egestion hypothesis. However, this putative saliva-inoculation event was not correlated with EPG waveforms.

The salivation-egestion hypothesis is, in some respects, a combination of the past and present hypotheses for vector inoculation of non- and semi-persistent pathogens, i.e., the egestion hypothesis [20] and the salivation hypothesis [28]. The salivation-egestion hypothesis may be applicable to all known or suspected cases of foregut-borne, non-circulative pathogen inoculation[27], such as *Maize chlorotic dwarf virus* by *Dalbulus* spp. leafhoppers [36] or *Cauliflower mosaic virus* by *Brevicoryne brassicae* L.

aphids, when given long AAPs [26]. The general idea that pathogens colonizing the precibarium can be loosened by a combination of mechanical turbulence and saliva in the precibarium, then egested out the stylet tips, seems applicable to multiple vector-pathogen systems. Because no experimental evidence contradicts the salivation-egestion hypothesis, it is likely that EPG was the breakthrough technology that solved the 70-year old mystery of the inoculation mechanism of *X. fastidiosa*.

The above-summarized basic research on the mechanism of *X. fastidiosa* inoculation has direct impact for applied problems in integrated pest management, also thanks to EPG. The sharpshooter X wave is a distinctive, readily recognized waveform that can be easily quantified. Present research in the Backus laboratory uses EPG to record vector behaviors on resistant vs. susceptible plants. Recent preliminary research (Backus, unpub. data) has shown that significantly fewer and shorter X waves are performed by insects on *X. fastidiosa*-resistant grapevines, compared with susceptible. Thus, it may be possible to select for grapevines that are resistant to vector inoculation behaviors, as well as *X. fastidiosa* growth and spread in the plant. Research is underway to eventually use quantitative analysis of X wave performance to develop an "inoculation behavior resistance index" that can be used by grape breeders to develop commercially viable, resistant grape with a diverse collection of *X. fastidiosa*-resistance traits. Thus, basic EPG research seamlessly evolves into applied research to solve real-world problems in management of vector-borne plant diseases.

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